Available online at www.sciencedirect.com
SCIENCE DIRECT®
Environmental Toxicology and Pharmacology 19 (2005) 85–98
ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY ETAP
www.elsevier.com/locate/etap

Gonadal histology and characteristic histopathology associated with endocrine disruption in the adult fathead minnow (*Pimephales promelas*)

Richard L. Leino[a,*], Kathleen M. Jensen[b], Gerald T. Ankley[b]

[a] *Department of Anatomy and Cell Biology, School of Medicine, University of Minnesota, Duluth, MN 55812, USA*
[b] *U.S. Environmental Protection Agency, Mid-Continent Ecology Division, 6201 Congdon Blvd., Duluth, MN 55804, USA*

Received 18 November 2003; accepted 6 May 2004
Available online 25 September 2004

Abstract

Examination of gonadal histopathology has been beneficial in understanding and assessing the effects of potential endocrine disrupting chemicals in fish and other organisms. The present study describes the normal gonadal histology of the fathead minnow (*Pimephales promelas*), a widely used test organism, reviews typical effects of endocrine disrupting chemicals with different modes/mechanisms of action on the histological structure of the ovaries and testes, and recommends methods for optimizing histopathological results.

Keywords: Fish; Ovary; Testis; Histology; Endocrine disruptors

1. Introduction

About 80,000 chemicals have been introduced into the environment within the last 50 years (Curtis and Skaar, 2002). There is mounting evidence that some of these chemicals may pose extensive, even global, threats to wildlife and humans (Curtis and Skaar, 2002; Fox, 2001; Vos et al., 2000). One of the most complex and heterogeneous classes of these compounds are those that might act upon the endocrine system, thereby adversely affecting reproduction and development. Endocrine disrupting chemicals (EDCs) may act in very small amounts and in subtle ways, affecting behavior and secondary sexual characteristics as well as the gonads themselves. It has been suggested that a variety of biomarkers and bioassays in the laboratory and in semi-field and field studies be used in determining the consequences of potential EDCs (Ankley et al., 2001; Gray et al., 2002; Parrott and Wood, 2002; van der Oost et al., 2003; Vos et al., 2000). With fish, which are an important potential target of environmental endocrine disruption, useful data may be obtained from biomarkers or assays ranging from molecular to ecosystem-wide levels of organization (van der Oost et al., 2003).

In 1996, the US Congress passed legislation requiring the US Environmental Protection Agency (USEPA) to develop a screening and testing program for specific classes of EDCs. In particular, the USEPA has focused on chemicals with the potential to adversely affect reproduction and/or development through alterations in processes controlled by estrogen, androgen, and thyroid hormones (USEPA, 1998). Proposed testing is divided into three phases, starting with ranking of all chemicals of potential concern for subsequent biological evaluations, followed by Tier 1 assays with the prioritized subset of chemicals and, finally, Tier 2 tests. The Tier 1 tests are proposed as comparatively rapid screens to focus the more extensive (and expensive) Tier 2 partial/full life-cycle evaluations (USEPA, 1998). Five prototype assays were recommended as Tier 1 tests; three of the tests use mammals (rats) as a model species, and the two remaining assays utilize amphibians (as a screen for thyroid function)

* Corresponding author. Tel.: +1 218 726 8879; fax: +1 218 726 6235.
E-mail address: rleino@d.umn.edu (R.L. Leino).

1382-6689/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.etap.2004.05.010

and fish, to detect EDCs with the potential to effect reproduction at any of a number of points in the hypothalamic-pituitary-gonadal (HPG) axis (Ankley et al., 1998; USEPA, 1998). The fish test is a short-term (21 days) reproduction assay with the fathead minnow (*Pimephales promelas*) that features a number of apical endpoints (survival, fecundity, fertility, hatch) in conjunction with endpoints diagnostic of alterations in specific aspects of endocrine function (Ankley et al., 2001). The diagnostic endpoints include alterations in secondary sex characteristics, changes in plasma concentrations of sex steroids or vitellogenin, and effects on gonadal histology. Various studies with compounds representative of different classes of EDCs have shown that the diagnostic endpoints can be quite specific with regard to identification of mechanism/mode of action (MOA). For example, androgenic chemicals cause morphological masculinization of reproductively mature female fathead minnows (Ankley et al., 2001, 2003), estrogenic compounds induce plasma vitellogenin in males (Ankley et al., 2001; Harries et al., 2000; Kramer et al., 1998), and xenobiotics that affect steroid metabolism alter circulating steroid concentrations in a predictable manner (Ankley et al., 2002). Effects of different EDCs on gonadal histopathology have not been as thoroughly characterized as some of these other diagnostic endpoints.

Histopathology has received increasing interest as an endpoint because histopathological changes are often the result of the integration of a large number of interactive physiological processes (van der Oost et al., 2003). Moreover, histopathological alterations allow effects to be visible at lower exposure levels than many other "integrative" endpoints, such as behavior changes and mortality (Wester et al., 2002). In the present study, we describe the normal histology of ovaries and testes of spawning-ready fathead minnows typical of those used in the protocol described by Ankley et al. (2001) and illustrate characteristic changes in these organs associated with EDCs. We also discuss some techniques for improving the resolving power of light microscopy of gonadal tissues by using methacrylate sections.

2. Methods and procedures for fathead minnow histopathology

2.1. Baseline studies

The fathead minnows used for these studies came from a culture, which has been maintained at the Duluth USEPA lab for more than 20 years. The fish are maintained at 25 °C under an 16:8 L:D photoperiod in a continuous flow of Lake Superior water and are fed newly-hatched brine shrimp ad libitum until they are about 1 month old, following which they receive thawed brine shrimp from frozen stocks. A detailed description of the culture situation relative to the baseline studies is presented by Jensen et al. (2001). Briefly, embryos from spawning adults are maintained in group cultures until they reach sexual maturity, typically within 5 months. Male and female fish are then paired and allowed to reproduce for 3–4 months before being replaced with new breeding pairs. Under these conditions, a clutch of eggs typically is produced every 3 days (Jensen et al., 2001).

To assess "normal" changes over time, pairs of fish in the culture unit were monitored daily for egg production, and sampled periodically to collect gonads representative of different gonadal stages during the 3-day reproductive cycle. The fish were deeply anaesthetized with MS-222, and gonads were gently excised form the body cavity, placed in cold 1% glutaraldehyde–4% formaldehyde in 0.1 M phosphate buffer, pH 7.4, for at least 24 h, dehydrated in graded ethanol solutions and embedded in methacrylate. The methacrylate blocks were sectioned at 2–3 μm in a step-wise fashion and stained with hematoxylin and eosin (with or without phloxin) or with methylene blue–azure A and basic fuchsin (see USEPA, 2002) and "*Tissue fixation, staining and sectioning*" below). Gonads used in the present study were typically removed from the body cavity to obtain weights for the gonadosomatic index and because other organs were often employed for analysis of gene expression or for biochemical studies such as measurement of aromatase activity. However, whole body sections, cut longitudinally with small fish and in cross-section with mature fish, were sometimes examined to compare gonads in situ with those that were excised.

2.2. Chemical exposures

An integrated evaluation of gonad histology in fathead minnows exposed to EDCs was achieved through consideration of samples generated at the Duluth USEPA lab using the design described by Ankley et al. (2001), as well as an analysis of the open literature for fathead minnow reproduction studies from other labs, conducted under relatively similar conditions. The experiments utilized chemicals representative of a range of MOA, including strong estrogen receptor agonists (17-β-estradiol, 17-α-ethinyl-estradiol; Miles-Richardson et al., 1999a; Lange et al., 2001), weak estrogen receptor agonists (nonylphenol, methoxychlor; Miles-Richardson et al., 1999b; Ankley et al., 2001), androgen receptor agonists (methyltestosterone, 17-β-trenbolone; Ankley et al., 2001, 2003; USEPA, 2002) androgen receptor antagonists (vinclozolin, flutamide; Makynen et al., 2000; Jensen et al., 2004) and an inhibitor of aromatase (Ankley et al., 2002).

The study by Lange et al. (2001) was a full-life cycle test. However, all of the other studies discussed were short-term, usually 21 days, reproduction assays conducted with spawning adults. The studies conducted at Duluth followed the standard protocol described elsewhere (USEPA, 2002), as well as in the aforementioned papers.

2.3. Tissue fixation, staining, and sectioning

Two approaches have commonly been employed in recent studies of fathead minnow histopathology of gonads and other tissues, one involving a traditional, paraffin-based, and

one a more specialized, methacrylate-based, histological procedure.

2.3.1. Paraffin-based procedure

2.3.1.1. Fixatives. Two fixatives have generally been used for paraffin-based studies of gonadal histology and histopathology in fathead minnows: (1) 10% neutral buffered formalin (McCormick et al., 1989), for example, (Roberts, 1978), and (2) Bouin's fluid (Roberts, 1978). Presumably Bouin's is used because of its capability for rapid fixation (4–6 h) and strong subsequent tissue staining. However, intracellular substances, such as granules and inclusions, are often poorly preserved with this fixative (Kiernan, 1990). Neutral buffered formalin gives better tissue preservation, but the gonads should ideally be fixed for 24 h or more.

2.3.1.2. Embedding. Paraffin embedding of fathead minnow gonads can be accomplished by standard methods, for example, as described in Kiernan (1990) and employing a graded series of ethanol solutions, an intermediate solvent, and infiltration in melted paraffin.

2.3.1.3. Sectioning and staining. Standard sectioning and staining procedures can be used with fathead minnow tissues. For example, sections may be cut at 5 μm and stained with hematoxylin and eosin. For ovaries, typically in our laboratory, a number of slides are made, each with one section from 500 μm deep into the longitudinally oriented organ and two sections from 1000 μm deep. Testes are sectioned in a similar manner, except that the sections are taken at 250 and 500 μm depths.

Advantages of paraffin techniques include: (1) a typical histology laboratory is set up to routinely process large numbers of samples, (2) more personnel are familiar with paraffin sectioning and staining procedures, (3) paraffin sections have been used in several recent EDC and other studies with fathead minnows, and (4) a wide variety of staining techniques are available, including some that involve immunostaining. Disadvantages include: (1) thicker sections allow less detail to be resolved, (2) several artifacts, e.g., due to tissue shrinkage during processing, are present, and (3) tissue samples have to be archived in other fixatives for high-resolution electron microscopy.

2.3.2. JB-4 methacrylate-based procedures

2.3.2.1. Fixatives. While the same fixatives (Bouin's, neutral buffered formalin) used in paraffin procedures may be employed, better tissue preservation is afforded by formaldehyde-glutaraldehyde fixatives when embedding in methacrylate. Both traditional electron microscopic fixatives, e.g., 2.5% glutaraldehyde–2% formaldehyde in 0.1 M phosphate buffer, or other fixatives, e.g., 1% glutaraldehyde–4% formaldehyde in 0.1 M phosphate buffer (Jensen et al., 2001) work well with fathead gonadal tissues. As with neutral buffered formalin, gonads should be fixed for at least 24 h prior to embedding.

2.3.2.2. Embedding. Embedding of the relatively small fathead minnow gonads can be accomplished rapidly in JB-4 methacrylate (Polysciences, Warrington, PA, USA) or in similar plastics, e.g., Immuno-Bed (Polysciences) for immunostaining, when compared to embedding in paraffin. A typical manual schedule for gonads (with tissue in vials on a rotator) is as follows:

25% ethanol	30 min
50% ethanol	30 min
75% ethanol	30 min
95% ethanol	30 min
100% ethanol	30 min
JB-4 solution A (catalyzed with 0.9 g catalyst/100 ml)	2 h

Embed (40 parts catalyzed solution A:1 part solution B; prevent contact with air during polymerization).

2.3.2.3. Sectioning and staining. Gonads are embedded in the same longitudinal orientation as with paraffin blocks. They are also sectioned in the same step-wise manner as paraffin blocks, but at a thickness of 2–3 μm. Staining of methacrylate sections can be accomplished as with paraffin procedures, but with slight modifications. Examples of two modified procedures that give similar results are presented below:

Hematoxylin and eosin (with phloxin)

Stain sections for 30–45 min with filtered Harris hematoxylin

Rinse with distilled water

Dry on a hot plate

Stain cooled slides for 1–2 min in saturated aqueous eosin containing 0.25% phloxin

Rinse in distilled water, dry on a hot plate, and coverslip

Basic fuschin and methylene blue–azure A

Stock basic fuschin:

1% basic fuchsin in 50% ethanol

Stock methylene blue–azure A in distilled water

1% azure A

1% methylene blue

1% borax

Dilute basic fuchsin 1:4–1:12 or more with distilled water

Stain 10–20 s and rinse with distilled water

Dilute methylene blue–azure A 1:2–1:4 or more with distilled water

Stain 10–20 s, rinse with distilled water, dry, and coverslip

Optimal staining times and stain dilutions will vary with the fixative used and with section thickness.

Advantages of the methacrylate technique include: (1) better fixation, less solvent-related extraction, and thinner sections allow for greater resolution of tissue and cellular details compared with paraffin sections (in fact, the superior resolution afforded by methacrylate sections may obviate the need for electron microscopy in many cases); (2) tissues embedded in methacrylate experience little shrinkage while those embedded in paraffin shrink 20% or more; (3) cutting distortion, an artifact related to tissue compression during sectioning, is greatly reduced in thin methacrylate sections; (4) staining of methacrylate sections, for example, with hematoxylin and eosin, is far simpler than paraffin sections, requiring no removal of embedding media or sequential hydration and dehydration steps; (5) when electron microscopy is desired, the archived tissue is already in a suitable (recommended) fixative; and (6) methacrylate sections have been used in several recent EDC studies. Disadvantages include: (1) some laboratories lack the facilities for methacrylate procedures, (2) hand processing of tissues is more expensive, (3) sectioning tissues larger than about 6 mm by 12 mm requires special wide glass knives, and (4) staining procedures must be modified for methacrylate sections. Given these considerations, methacrylate embedding of tissues fixed for at least 24 h in formaldehyde–glutaraldehyde is the method of choice in our laboratory: the greater resolution and fewer artifacts seen in methacrylate sections make it worthwhile to establish a 'methacrylate' facility in histopathology laboratories where EDC studies are performed.

2.4. Evaluating EDC-induced histological changes in fathead minnow gonads

2.4.1. Ovaries

Gonadal staging, i.e., as described for ovaries in Table 1, is a fundamental method for discerning effects of EDCs on these organs. For example, if ovaries of control fish are stage 4, and those of EDC-exposed fish are stage 3, EDC exposure has resulted in a major effect on ovarian development. Gonadal staging is recommended as the first step for the histological evaluation of EDC effects.

Other methods used to describe and evaluate EDC effects on gonads are still evolving based on the kinds of histopathological changes that are being observed. Perhaps the most important of the published methods attempt to quantify a histological change, as in counting the numbers of follicles in various stages of development (Miles-Richardson et al., 1999a,b; Smith, 1978). Counting of follicles provides more information on whether the percentage of particular stages is abnormally low or high than does staging. For example, treatment of fathead minnows with 10 nM of estradiol for 14 days resulted in a greatly increased percentage of primary follicles and a decreased percentage of mature follicles (Miles-Richardson et al., 1999a). A recommended method involves counting 100 follicles from sections taken from between 500 μm into the ovary and its midline, and calculating the percentage of each follicular stage present. A similar method has also been employed to assess the severity of oocyte atresia (McCormick et al., 1989). That investigation determined that a critical mean percentage of 20% atretic follicles affected spawning success in groups of fathead minnows exposed to acidified water. This percentage is higher than the 10–12% atresia occasionally seen in normally spawning females (see below).

2.4.2. Testes

As is the case for ovaries, testicular staging (Table 2) represents the initial step in evaluating the histological effects of EDCs. However, as with ovaries, testicular staging will likely reveal only EDC effects that profoundly influence testicular maturation. Certain quantitative methods have been employed to describe more subtle changes in testicular histology (Smith, 1978) including: (1) *Percentage of testicular stages present*. Determination of the percentage of each testicular stage present, such as primary and secondary spermatogonia and spermatocytes, can provide information as to whether any of these stages has an atypical distribution. Unlike ovaries, the relatively small and more numerous testicular germ cells are difficult to count properly without an ocular grid or similar device. Smith (1978) employed an ocular grid to evaluate testicular developmental stages, counting 100 cells in each of three sections per fish. It is important to include different regions from the same testes because testicular histology sometimes varies from one region to another, in contrast to ovaries, which

Table 1
Histological stages of fathead minnow ovarian development

1. Primary growth	Oogonia and primary oocytes 1a. Oocytes in nests; small cytoplasmic volume (Fig. 3) 1b. Oocytes larger, out of nests, surrounded by follicle cells; many pleiomorphic nucleoli bordering the nuclear envelope (Fig. 4)
2. Cortical alveolus	Appearance of cortical alveoli and scattered small lipid droplets (Fig. 5)
3. Early vitellogenic	Appearance of yolk bodies: initially few and small; ultimately many and variably-sized; centrally located germinal vesicle is round to oval with several peripheral nucleoli (Fig. 6)
4. Late vitellogenic	Germinal vesicle loses nucleoli, moves towards the periphery and breaks down; yolk bodies frequently fill the entire center of the oocyte and a germinal vesicle may not be evident (Fig. 6)
5. Mature/spawning oocyte	Germinal vesicle breakdown complete; yolk bodies fuse and may become larger than cortical alveoli

Table 2
Histological stages of fathead minnow testicular development (see Fig. 11)

1. No developing and only resting germ cells present	
2. Spermatogonia	2a. Primary spermatogonia: large cells near edges of tubule; have a lightly staining nucleus with a prominent nucleolus 2b. Secondary spermatogonia: clusters of medium-sized cells with a round, lightly basophilic nucleus; cluster or cyst is the result of several mitotic divisions of a primary spermatocyte
3. Spermatocytes	3a. Primary spermatocytes: smaller cells with smaller, more basophilic nuclei than spermatogonia: will undergo meiosis I to produce secondary spermatocytes 3b. Secondary spermatocytes: small cells with smaller, more basophilic nuclei than primary spermatocytes: will undergo meiosis II to produce spermatids
4. Spermatids and some spermatozoa in lumen of seminiferous tubule; small tubule lumen	Spermatids have a small, intensely basophilic nucleus; they mature into spermatozoa
5. Abundant sperm in an expanded lumen	(Figs. 13 and 14)

seem to have a rather uniform distribution of oocyte stages throughout. (2) *Tubule diameter*. Certain EDCs may enhance or decrease sperm production. Histologically, this may manifest itself as an enlargement or reduction in the mean diameters of seminiferous tubules. Smith (1978) and Gimeno et al. (1998) described methods to measure and quantify changes in tubule diameters and relate these changes to sperm production. Again, due to variations in different areas of the tissue, tubule diameters should be measured in several testicular regions.

With zebrafish, another species widely used in EDC studies, similar and novel measurements, most involving computerized morphometry of testes have shown promise in revealing testicular changes upon exposure to EDCs (van den Belt et al., 2002; van der Ven et al., 2003). These measurements included the size and numbers of various cyst stages, anomalies of meiosis I in cysts, Sertoli and Leydig cell hyperplasia or hypertrophy, and changes in testicular interstitial volume primarily due to EDC-induced deposition of vitellogenin. With some adaptation, these approaches could be used with fathead minnows, as well.

2.4.2.1. Special considerations for testes. Electron microscopy. Some EDC-induced histopathological changes in testes are difficult to study by light microscopy because of the small sizes of the affected cells. This is particularly evident with Sertoli cells, which may undergo major EDC-induced changes in morphology (Miles-Richardson et al., 1999a,b). Those investigators used electron microscopy to describe changes in Sertoli cells in conjunction with spermatocyte necrosis. Spermatocyte necrosis appears to be a common result of EDC exposure (Miles-Richardson et al., 1999a,b), and electron microscopy is beneficial for supplementing light microscope-based descriptions of this pathology. Electron microscopy may also be useful when studying the effects of EDCs on another small testicular cell, the Leydig cell. Although it is reasonable to assume that EDCs may affect Leydig cells, no studies have yet been published on this subject for fathead minnows.

Testicular atrophy and ova–testes. Major changes in the testes, sometimes even making them difficult to identify in histological sections, can be produced by EDÇs. For example, Lange et al. (2001) reported ova–testes and frank testicular atrophy in fathead minnows after long-term exposures to ethylenestradiol. More work needs to be done on describing the major histopathological events leading to testicular atrophy.

3. Results and discussion

3.1. Gonadal histology of reproductively-mature fathead minnows

3.1.1. Ovaries

3.1.1.1. General structure. The ovaries are paired organs that, when mature, occupy much of the abdominal cavity ventral to the swim bladder (Grizzle, 1979). They are suspended by a mesentery, the mesovarium, that is attached to the dorsal wall of the abdominal cavity, the dorsal part of the swim bladder near the kidneys and to the wall of the abdominal cavity slightly lateral to them. The mesovarium

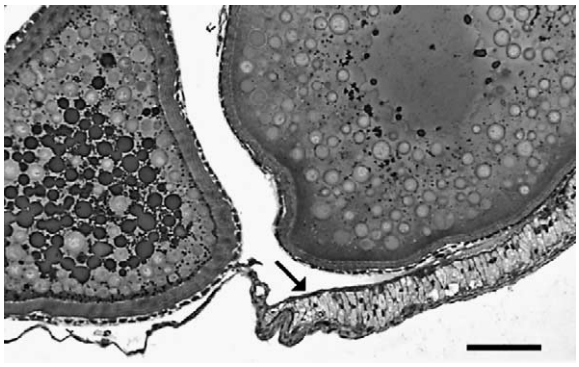

Fig. 1. Section through ovary showing part of the tunica albugenea. The lining epithelium varies from columnar (arrow) to squamous. Micrographs are from methacrylate sections stained as described with hematoxylin and eosin. Bar = 88 μm.

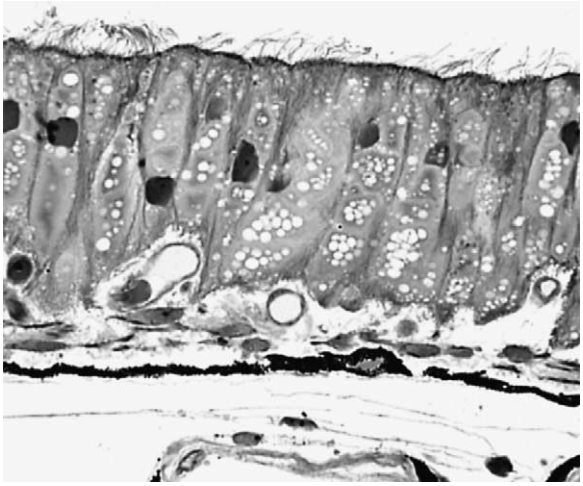

Fig. 2. Higher magnification of the columnar epithelium of the tunica albugenea. The epithelium appears to be ciliated. Bar (see Fig. 1) = 19 μm.

is continuous with the peritoneum that covers the ovary proper. The peritoneum is composed of a mesothelium and an underlying layer of connective tissue, the tunica albuginea. In fathead minnows, rodlet cells are found among the squamous cells of the mesothelium, and eosinophilic granular cells, melanocytes, smooth muscle, blood vessels, and nerves are common within the connective tissue layer. The visceral border of the tunica albuginea is lined with an epithelium that is squamous ventrally and columnar dorsally (Figs. 1 and 2). The columnar cells appear to be ciliated. Ovigerous lamellae extend from the tunica albuginea toward the center of the ovary, dividing it into lobules that contain the oogonia and developing oocytes (Grizzle, 1979).

3.1.1.2. Gametogenesis. Fathead minnows may spawn in as little as four months after hatching (Jensen et al., 2001). Little is known about gonadal development during this time, but oogonia are identifiable in ovaries of very young juveniles and oocytes are present in older juveniles (Grizzle, 1979).

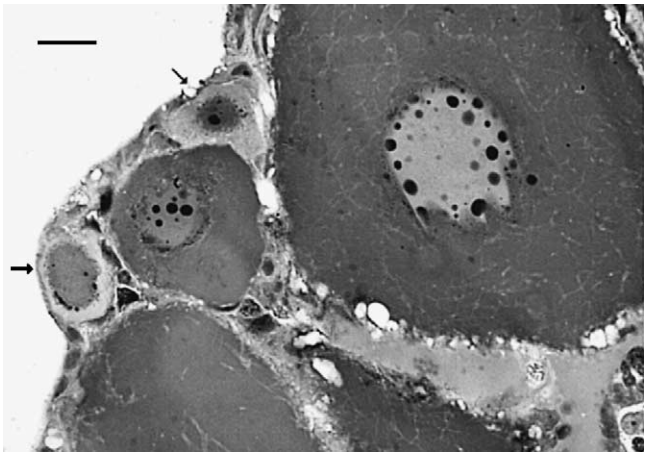

Fig. 3. Oogonia or small primary oocytes (arrows) in nest with larger primary oocytes. Bar = 10 μm.

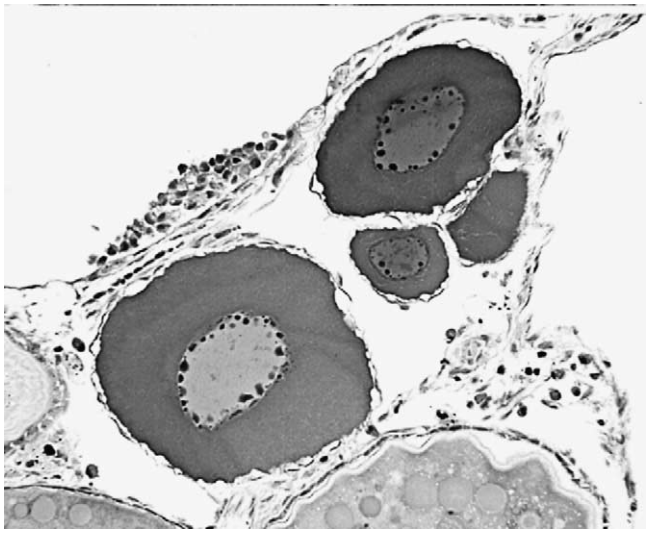

Fig. 4. Late primary growth oocytes of various sizes. Late primary oocytes are surrounded by squamous follicle cells. Bar (see Fig. 3) = 27 μm.

However, since fathead minnows are fractional spawners, all oocyte developmental stages are seen in a mature ovary: (1) oogonia, (2) primary growth stage oocytes, (3) cortical alveolus stage oocytes, and (4) early and late stage vitellogenic oocytes.

Oogonia. Oogonia (and/or the smallest oocytes present; see Selman et al. (1993) are small cells (12–20 μm in diameter in methacrylate sections) occurring in groups or nests along with similar-sized and larger primary oocytes (Fig. 3). Oogonia have a large nucleus with a few variably sized nu-

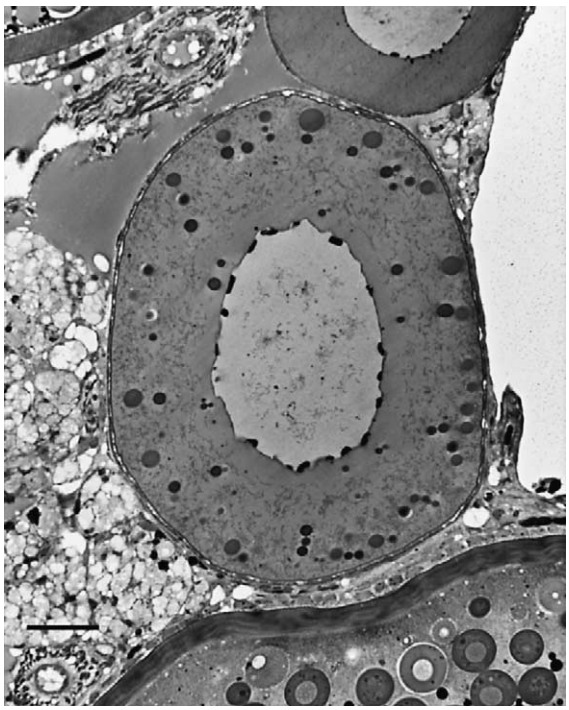

Fig. 5. Cortical alveolus stage oocyte. Bar = 35 μm.

cleoli and a relatively narrow rim of cytoplasm. They are not surrounded by follicular cells.

Primary growth oocytes. Early primary growth oocytes (12–35 μm diameter) are often seen next to other primary oocytes (Fig. 3). They are still in nests and not completely surrounded by follicular cells, which are also present in the nests. Early primary oocytes have a round or oval nucleus with a few variably sized nucleoli. The cytoplasm contains no cortical alveoli or yolk bodies. Late primary oocytes (35–170 μm diameter) have exited from cell nests and become completely surrounded by squamous follicle cells (Fig. 4). Both cytoplasmic and nuclear (germinal vesicle) volumes increase considerably during the primary growth phase, as do the numbers of nucleoli, which tend to lie close to the nuclear envelope.

Cortical alveolus oocytes. Cortical alveolus oocytes (170–425 μm diameter) are characterized by the appearance of cortical alveoli ("yolk vesicles") and, in some fishes, small lipid droplets, in the cytoplasm. Fathead minnow oocytes typically have only a few lipid droplets, based on staining with Sudan (lipid soluble; (Bronner, 1975)) dyes. In early cortical alveolus oocytes, it is possible to observe only one or two cortical alveoli; larger oocytes have many cortical alveoli distributed throughout the cytoplasm (Figs. 5 and 6). The centrally positioned germinal vesicle is oval during this stage and has numerous peripherally located nucleoli of various sizes. The vitelline envelope (zona radiata) is clearly visible in even the smallest cortical alveolus oocytes, and the follicle cells are squamous in early-, and more cuboidal in late, cortical aveolar oocytes.

Vitellogenic oocytes. The initiation of vitellogenesis forming vitellogenic oocytes (425–1070 μm diameter) represents the next developmental stage and is characterized by the accumulation of eosinophilic yolk bodies in the ooplasm. In fathead minnows the yolk bodies are also weakly sudanophilic (Leino, 2003, unpublished). At first the yolk bodies are much smaller than cortical alveoli and mostly dispersed among them, especially in the perinuclear cytoplasm (Fig. 6). As the oocyte grows, the yolk bodies become larger and more numerous and displace the cortical alveoli, pushing them to the periphery of the oocyte (Figs. 6 and 7). In the late vitellogenic oocyte (800–1070 μm diameter) the germinal vesicle also appears to move toward the periphery of the oocyte and then to disappear entirely when the oocyte approaches maturity (Fig. 6). In vitellogenic oocytes the vitelline envelope thickens and becomes striated due to the great numbers of pore channels that penetrate through it. The follicle cells are

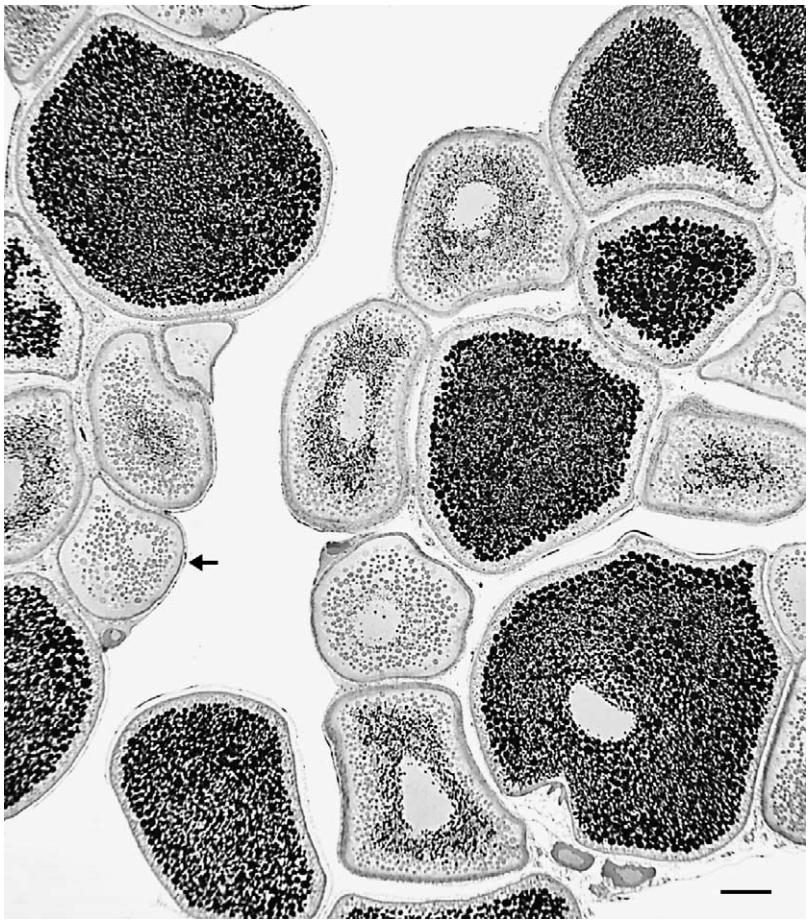

Fig. 6. Part of ovary containing early and late vitellogenic oocytes with densely stained yolk bodies. Note single cortical alveolus stage oocyte (arrow). Bar = 165 μm.

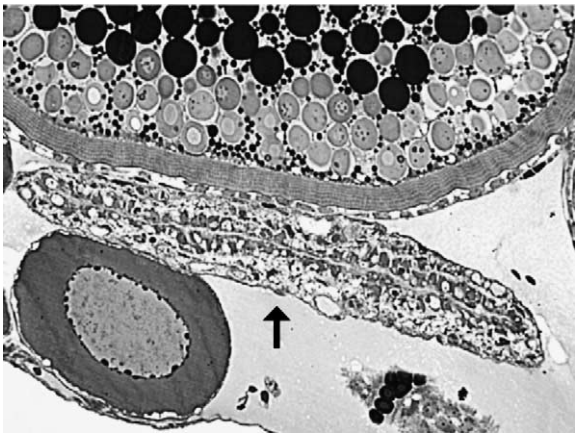

Fig. 7. Vacuolated post-ovulatory follicle (arrow) from a day 1 ovary. Bar (see Fig. 8) = 40 μm.

cuboidal with a large nucleus and a prominent round nucleolus. External to the follicular layer lies a thin basal lamina and a theca consisting of squamous thecal cells, capillaries, and a thin connective tissue stroma. In the most mature oocytes observed in tissue sections some yolk bodies coalesce and become larger than the cortical alveoli but remain numerous rather than joining into a single yolk mass.

3.1.1.3. Staging. Staging of ovaries of fractional spawners, such as the fathead minnow, has generally been based upon the maturest oocytes present in the histological section (Jensen et al., 2001; Leino and McCormick, 1997; Selman and Wallace, 1986; Selman et al., 1993; Shimizu, 1997). Such a classification, based on the studies above, is presented in Table 1.

In fathead minnows, stage 5 oocytes are rarely observed unless females are sampled while spawning. In other words, late stage 4 oocytes are apparently only hours away from being ovulated.

3.1.1.4. Ovarian stages in normal, reproducing females. With optimal laboratory spawning conditions (e.g., 16 h photoperiod, 25 °C water) most female fathead minnows spawn every 3–4 days (Jensen et al., 2001). Some, however are daily spawners, at least for a period of time. Others spend many days between spawnings. The following description considers variations in ovarian histology during a typical spawning cycle.

- Day 0: Day 0 ovaries were sampled within about 8 h after spawning, which usually takes place in the early morning. These ovaries had returned to late stage 3. Post-ovulatory follicles ("corpora lutea") are numerous. In some ovaries most of these follicles are collapsed while in others the follicle lumen is open (Figs. 8 and 9). In goldfish, post-ovulatory follicles have lumens for at least 10 h after ovulation, but the follicles collapse by 30 h post-ovulation (Nagahama et al., 1976). This phenomenon may occur more rapidly in fathead minnows.

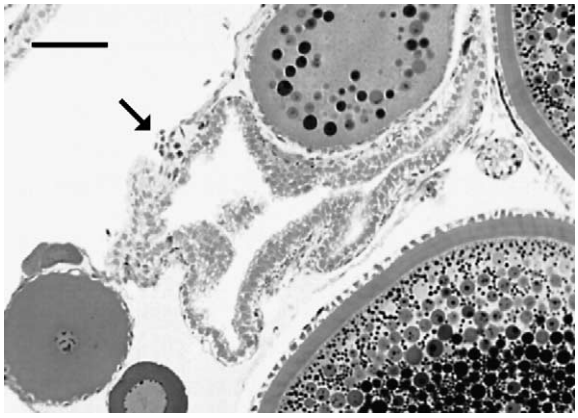

Fig. 8. Post-ovulatory follicle ("corpus luteum", arrow) with an open lumen from a day 0 ovary (see text). Bar = 60 μm.

- Day 1: Ovaries sampled on day 1 after spawning have progressed to stage 4. The post-ovulatory follicles tend to be smaller and have thinner walls than at day 0. Some are vacuolated and appear to be breaking down (Fig. 7).
- Day 2: Day 2 ovaries tend to be at late stage 4. Post-ovulatory follicles are often difficult to identify. Those that are present are small and highly vacuolated.
- Day 3 (spawning): Ovaries reach stage 5–spawning ready.

3.1.1.5. Atretic follicles in normal, reproducing females. If an EDC affects ovarian development and/or spawning, a logical histological feature to assess is an increase in numbers of atretic follicles (pre-ovulatory atretic follicles (POAFs)). In fathead minnows (McCormick et al., 1989) follicular atresia is initially indicated by breaks or gaps that appear in the vitelline envelope (Fig. 10). Subsequently, the nucleus and yolk material begin to degenerate, and absorption of yolk results in the follicle becoming less densely stained. Ultimately, a shell of follicular cells in various stages of degeneration surrounds a loose mass of oocyte residue (McCormick et al., 1989) before the structure collapses and forms a small cell mass that then disappears. Follicular atresia in experimental populations of reproducing fathead minnows is gen-

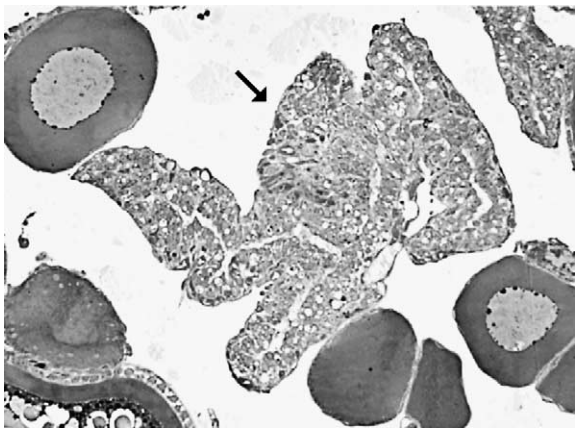

Fig. 9. Collapsed post-ovulatory follicle (arrow) from a day 0 ovary. Bar (see Fig. 8) = 60 μm.

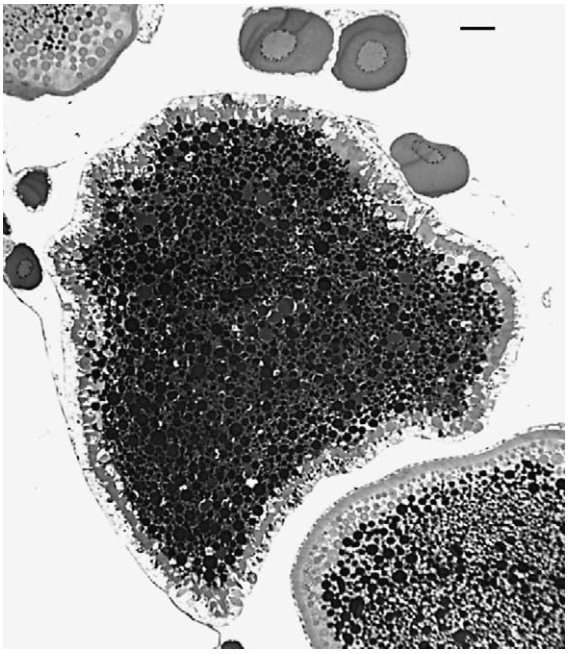

Fig. 10. Pre-ovulatory atretic follicle in early stage of atresia; note breakdown of zona radiata. Bar = 120 μm.

erally at a low level (Jensen et al., 2001). McCormick et al. (1989) reported a mean atresia level (in controls) of 1.6% (range 0–11.6%, n = 10) in their experiments, and Miles-Richardson et al. (1999b) reported a level of 4.6% (range 0–12%, n = 7). Although most females have a very low incidence of POAFs, a few specimens have high levels. For example, in the McCormick et al. (1989) study, nine ovaries had atresia levels of 0–1.3% while only one ovary had a substantially higher level of 11.6%. Out of 27 "control" ovaries examined for the present document, only three had relatively high levels of atresia. Examination of these ovaries showed them to be otherwise histologically normal, at prespawning late stage 4, and with atresia mostly or entirely of the maturest follicles (Fig. 10). The histology is suggestive of the female "missing" a spawning opportunity. Overall, follicular atresia in the 10–12% range may be part of a normal process in some (or perhaps many) fathead minnows some time during the long spawning period. No obvious background pathology, including testes–ova, were observed in our samples.

3.1.2. Testes

3.1.2.1. General structure. The testes are a pair of elongated white organs situated in the dorsal body cavity. Like the ovaries they are suspended by a peritoneal mesentery. Peritoneum covers the testes and consists of a layer of simple squamous epithelium and a thin connective tissue capsule, the tunica albuginea (Grizzle, 1979). Connective tissue septa that separate the seminiferous tubules are continuous with the tunica albuginea. The seminiferous tubules contain the germinal epithelium that ultimately gives rise to spermatozoa (Fig. 11). In mature testes spermatozoa are present in the lumens of seminiferous tubules and of the dorsally situated ampullae that are similar to seminiferous tubules, but lack germinal epithelium (Grizzle, 1979). Ampullae empty into the ductus deferens.

In addition to the germ cells, two other principal cell-types are present in the testes: Sertoli cells and interstitial cells of Leydig. Sertoli cell bodies, the part of the cell that contains the nucleus, are small and difficult to locate. At high magnification these cell bodies are often triangular shaped structures situated near the outer rim of the seminiferous tubule (Fig. 12). The elongate euchromatic nucleus often exhibits a single nucleolus. Processes of a Sertoli cell envelope a cluster of developing germ cells derived from a single primary spermatogonium to form a cyst. These cytoplasmic processes are not usually visible with the light microscope (Grizzle, 1979). Numerous polyhedral-shaped Leydig cells are found, usually in groups, in connective tissue spaces between seminiferous tubules (Fig. 12). They typically have an oval heterochromatic nucleus and a narrow rim of cytoplasm.

3.1.2.2. Gametogenesis and staging. The germinal epithelium of fathead minnows has an apparently random distribution of spermatogonia along the entire length of the tubule, the so-called "unrestricted" type of testis (Grier, 1981; Jensen et al., 2001). Spermatogonia are located in small peripheral cysts in the tubule; these cysts enlarge and extend toward the tubule lumen as spermatogenesis proceeds. Five stages of germ cell development are readily identified in the fathead minnow: (1) primary spermatogonia, (2) secondary spermatogonia, (3) primary spermatocytes, (4) secondary spermatocytes, and (5) spermatids and spermatozoa (Grizzle, 1979; Jensen et al., 2001; Smith, 1978). The presence or absence of these stages in a histological section, then, can be used to judge the state of testicular maturity. However, a better idea of how many sperm are being produced may be obtained by considering the relative size and sperm content of the seminiferous tubules (Gimeno et al., 1998; Leino et al., 1990; Smith, 1978), as in Table 2.

3.1.2.3. Testicular changes in normal, reproducing males. During a typical 3-day spawning cycle the testes do not seem to regress to an earlier stage as ovaries do. Examination of testes at 0, 1, 2, and 3 days after spawning revealed, instead, that just after spawning certain seminiferous tubules or regions of these tubules became largely depleted of sperm and had a thin germinal epithelium. Other tubules, however, had a thick germinal epithelium or abundant sperm or both. Apparently, sperm production is unlikely to be diminished during normal laboratory spawning intervals (Fig. 13).

3.2. Endocrine disrupting chemicals and gonadal histopathology

Several recent studies involving exposure of fathead minnows to waterborne EDCs have included an examination of gonadal histopathology. Most, but not all, of these studies considered effects on both ovaries and testes.

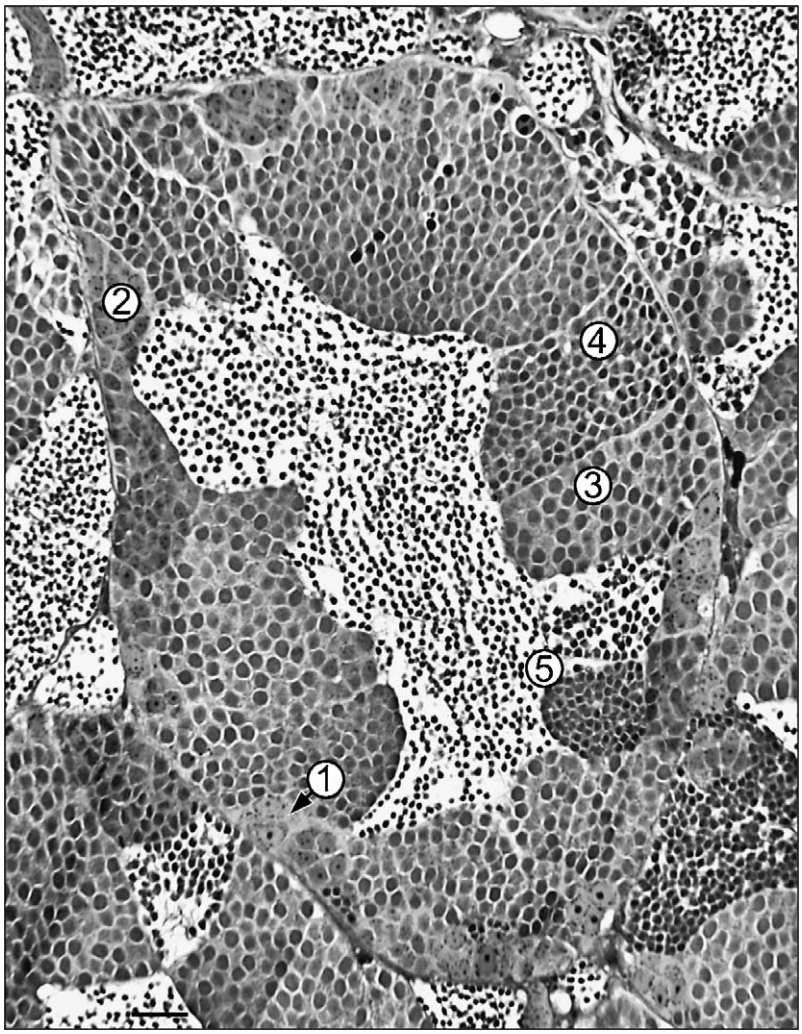

Fig. 11. Section of a seminiferous tubule showing various developmental stages: (1) primary spermatogonia, (2) secondary spermatogonia, (3) primary spermatocyte, (4) secondary spermatocyte, and (5) spermatids, late cyst above and early cyst beneath. Bar = 17 μm.

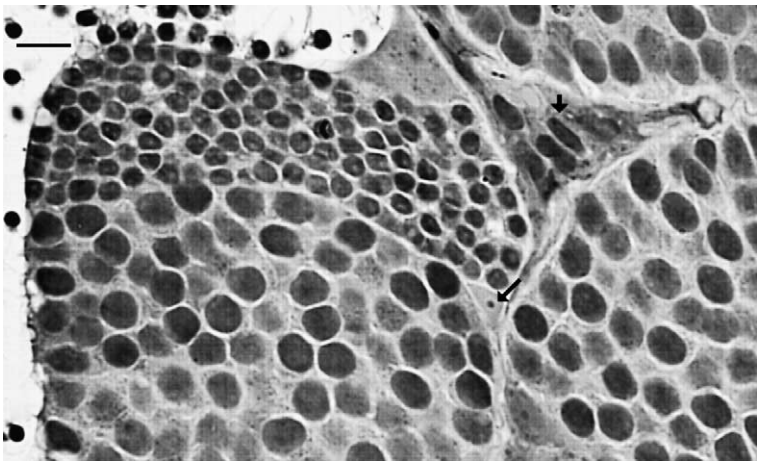

Fig. 12. Sertoli cell (long arrow) and interstitial cells of Leydig (short arrow) in testis. Bar = 6.5 μm.

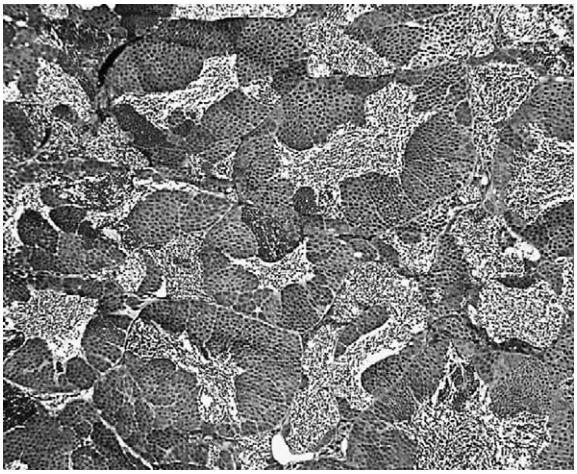

Fig. 13. Stage 5 testis with thick germinal epithelium and sperm-filled lumens. Bar (see Fig. 14) = 140 μm.

3.2.1. Strong estrogen receptor agonists

The strong estrogen receptor agonists (17-β-estradiol [E2], ethinylestradiol [EE2]) examined to date have similar effects on fathead gonads (Kramer et al., 1998; Lange et al., 2001; Miles-Richardson et al., 1999a). Both E2 and EE2 seem to interfere with egg production. Ovaries of treated fish exhibit fewer mature, and more atretic, follicles. Kramer et al. (1998) and Miles-Richardson et al. (1999a) noted that E2 treatment resulted in a sustained increase in plasma vitellogenin in females. They suggested that sustained abnormally high vitellogenin levels interfere with final maturation and release of oocytes from the ovary, possibly by inhibiting gonadotropin II release by the pituitary.

Strong estrogen receptor agonists also affect the testis. Testicular changes vary, depending on dosage, from slight degeneration, especially involving spermatozoa, to frank atrophy and appearance of testes–ova. Moreover, Miles-Richardson et al. (1999a) reported that a dose-dependent proliferation of Sertoli cells containing remains of spermatozoa accompanied degeneration of spermatozoa.

The aforementioned studies concluded that the reduced reproductive success associated with exposure to strong estrogen agonists is likely due to effects on both ovaries and testes.

3.2.2. Weak estrogen receptor agonists

Nonylphenol and methoxychlor are putative weak estrogen agonists that have been tested in fathead minnow exposures that included a gonadal histopathology component.

No histological changes were observed in ovaries of minnows exposed to 0.5–3.4 μg/l of nonylphenol, or similar concentrations of its parent compound, nonylphenol ethoxylate, for 42 days (Miles-Richardson et al., 1999b). Although egg production was increased at some nonylphenol concentrations, this would not necessarily be apparent on histological examination of the ovaries.

High (3.6 μg/l) concentrations of methoxychlor resulted in increased follicular atresia in some females (Ankley et al., 2001). This finding is consistent with a substantial reduction in numbers of eggs produced at this high methoxychlor concentration during a 21-day exposure.

In testes, exposure to 1.1 or 3.4 μg/l nonylphenol resulted in significant necrosis of germ cells and spermatozoa accompanied by hyperplasia and hypertrophy of Sertoli cells containing germ cell remnants (Miles-Richardson et al., 1999b). In contrast, methoxychlor had no discernable effect on testis histology despite the finding that plasma of males exposed to this agent had significantly decreased levels of testosterone and 11 ketotestosterone.

Taken together, the weak estrogen receptor agonists studied so far have exhibited effects on the histology of either ovaries (methoxychlor) or testes (nonyphenol), or neither organ (nonyphenol ethoxylate), suggesting that there may be different MOA for different weak agonists.

3.2.3. Strong androgen receptor agonists

Ankley et al. (2001) examined the effects of the strong androgen agonist methyltestosterone on fathead minnows during a 12 days flow-through study. Even at the lowest concentration tested (0.2 mg/l), spawning promptly ceased. Examination of the ovaries of treated fish after the 12-day exposure revealed no post-ovulatory follicles. Instead there were numerous pre-ovulatory atretic follicles as in Fig. 10, and maturation of younger follicles was suppressed. Thus, ovaries of fish exposed to methyltestosterone may rapidly revert to an earlier developmental stage.

Methyltestosterone-exposed testes differed from controls in that they appeared to be stimulated to near exhaustion of germinal epithelial stages. The germinal epithelium was much thinner, and “spermatogenic activity” more scattered than usually observed in control testes (as in Fig. 14, Ankley et al., 2001).

Recently, Ankley et al. (2003) reported results very similar to those in the methyltestosterone study were observed in fathead minnows exposed to the androgenic growth promoter 17-β-trenbolone. Specifically, trenbolone exposure reduced (0.05 μg/l) or abolished (0.5 μg/l) egg production. The ovaries, even at the lower trenbolone concentration, were at an earlier developmental stage than in unexposed females. Many of the vitellogenic and atretic follicles from exposed fish had an unusual appearance in that yolk deposition was low for their size, apparently reflecting trenbolone’s propensity to reduce plasma vitellogenin concentrations.

As with methyltestosterone, the testes of trenbolone (0.5 μg/l)-exposed fish exhibited thinned germinal epithelia and scattered spermatogenic activity as in Fig. 14.

3.2.4. Androgen receptor antagonists (or antiandrogens)

Vinclozolin, a fungicide that exhibits anti-androgenic properties in mammals, was tested with fathead minnows in a 21-day study (Makynen et al., 2000). The major histological finding was that, at the highest concentration tested

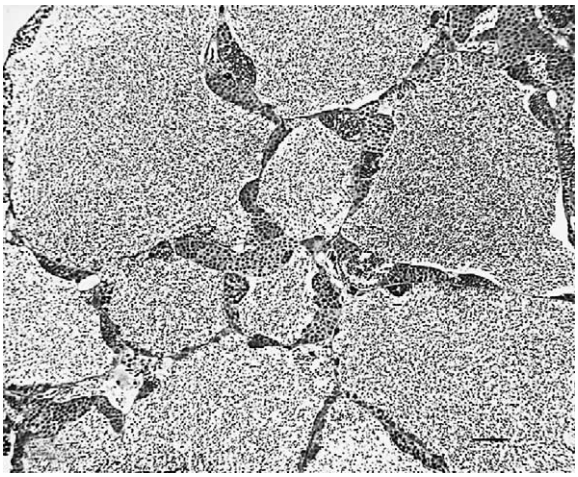

Fig. 14. Stage 5 testis with thin germinal epithelium and expanded lumens commonly observed with exposure to androgen agonists such as trenbolone. Bar = 140 μm.

(700 μg/l), oocyte diameters were significantly reduced. That is, the ovaries exhibited retarded maturation, especially regarding yolk deposition in oocytes. Paradoxically, there were no observable effects of the putative antiandrogen on the testes at any concentration tested.

Flutamide, another antiandrogen, decreased fecundity in the fathead minnow in a concentration-dependent fashion (Jensen et al., 2004). At a concentration of 651 μg/l, egg production was only about 25% of control values. Examination of the ovaries of flutamide-exposed fish revealed that most or all ovaries in each group were classified as stage 4–spawning ready. Subtle changes among treatments included: (1) more early-stage (1 and 2) follicles and (2) more atretic follicles in some ovaries of treated fish. These factors could indicate decreased egg production via either a delay in oocyte maturation resulting in fewer eggs being produced in a given time, or a greater than average number of follicles undergoing resorption before reaching maturity.

No changes that would seem to alter sperm production were observed in testes of flutamide-exposed fish, although there were a few cysts with variable numbers of degenerating cells (probably secondary spermatogonia and/or primary spermatocytes) with pyknotic nuclei in the seminiferous tubules of some testes (Fig. 15).

3.2.5. Steroid metabolism inhibitor

Fadrozole is an aromatase inhibitor that blocks the conversion of testosterone to E2 in fish (Afonso et al., 2000; Ankley et al., 2002). In a 21-day reproduction study with fathead minnows exposed to fadrozole, spawning was greatly reduced at the lowest test concentration (1.4 μg/l), and completely arrested at two higher concentrations (7.3 and 57 μg/l) (Ankley et al., 2002). Histologically, the ovaries exhibited (1) near (lowest concentration) or complete (higher concentrations) absence of post-ovulatory follicles, (2) increased numbers of pre-ovulatory atretic follicles, and (3) regression to an earlier developmental stage (higher concentrations). There were no differences in the testicular stage (4-mature with plentiful sperm) between the control and the treatment groups. However, all males from the two higher concentration groups had more sperm in their seminiferous tubules than did controls and the low concentration group.

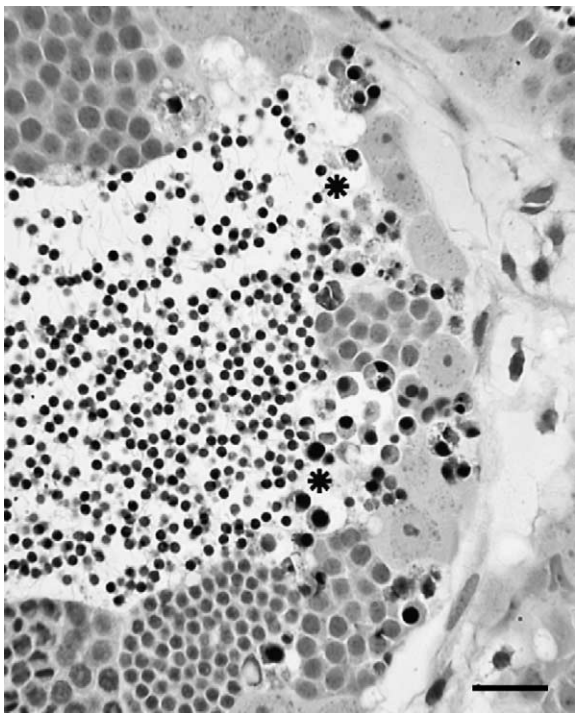

Fig. 15. Section of seminiferous tubule of male exposed to the antiandrogen flutamide for 21 days showing cysts with damaged and pyknotic cells (asterisks). Bar = 13.5 μm.

In fathead minnows, the aromatase inhibitor fadrozole, judging by histological criteria, seems to shut down spawning by preventing maturation of oocytes, while having no inhibitory effect on testes. The striking accumulation of sperm in testes from the two higher concentration groups could be due to stimulation of sperm production or to greater sperm storage due to lack of spawning.

3.3. Conclusions

In this work we describe “normal” gonadal histology in reproductively active female and male fathead minnows. We also describe histopathology associated with EDC exposures and propose that histopathological procedures using tissues embedded in methacrylate offer certain advantages over conventional paraffin techniques. In fathead minnows, studies of compounds representative of various classes of EDCs have revealed that, while they may have a different MOA, exposure to these chemicals often results in similar pathological changes in the gonads. This is notably the case with ovaries where exposure to estrogen receptor agonists, androgen receptor agonists, androgen receptor antagonists, and steroid metabolism inhibitors affect oocyte development

and maturation resulting in the reduction or cessation of ovulation. Effects of EDCs on testes seem to be more variable, for example, resulting in degenerative changes (estrogen receptor agonists) or possible hyper-production of sperm (androgen receptor agonists, aromatase inhibitors). Gonadal histopathology is a useful tool in EDC studies and may provide information on the MOA of EDCs that is sometimes counterintuitive and unexpected.

Acknowledgements

We thank our many colleagues of the Duluth EPA lab who have participated in EDC studies with the fathead minnow that produced the samples described/discussed herein This paper has received clearance for publication in accordance with official EPA policy. Mention of trade names does not indicate endorsement by the Federal government.

References

Afonso, L.O.B., Iwama, G.K., Smith, J., Donaldson, E.M., 2000. Effects of the aromatase inhibitor Fadrozole on reproductive steroids and spermiation in male coho salmon (*Oncorhynchus kisutch*) during sexual maturation. Aquaculture 188, 175–187.

Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J., Makynen, E.A., 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20, 1276–1290.

Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., Henry, T.R., Denny, J.S., Leino, R.L., Wilson, V.S., Cardon, M.C., Hartig, P.C., Gray, L.E., 2003. Effects of the androgenic growth promoter 17-beta-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. Environ. Toxicol. Chem. 22, 1350–1360.

Ankley, G.T., Kahl, M.D., Jensen, K.M., Hornung, M.W., Korte, J.J., Makynen, E.A., Leino, R.L., 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). Toxicol. Sci. 67, 121–130.

Ankley, G.T., Mihaich, E., Stahal, R., 1998. Overview of a workshop on screening methods for detecting potential (anit-)-estrogenic/androgenic chemicals in wildlife. Environ. Toxicol. Chem. 17, 68–87.

Bronner, B., 1975. Simultaneous demonstration of lipid and starch in plant tissues. Stain Technol. 50, 1–4.

Curtis, C., Skaar, T., 2002. Ubiquitous and dangerous. Our Planet, 24–26.

Fox, G.A., 2001. Effects of endocrine disrupting chemicals on wildlife in Canada: past, present, future. Water Qual. Res. J. Can. 36, 233–251.

Gimeno, S., Komen, H., Jobling, S., Sumpter, J., Bowmer, T., 1998. Demasculinisation of sexually mature male common carp, *Cyprinus carpio*, exposed to 4-tert-pentylphenol during spermatogenesis. Aquat. Toxicol. 43, 93–109.

Gray Jr., L.E., Ostby, J., Wilson, V., Lambright, C., Bobseine, K., Hartig, P., Hotchkiss, A., Wolf, C., Furr, J., Price, M., Parks, L., Cooper, R.L., Stoker, T.E., Laws, S.C., Degitz, S.J., Jensen, K.M., Kahl, M.D., Korte, J.J., Makynen, E.A., Tietge, J.E., Ankley, G.T., 2002. Xenoendocrine disrupters-tiered screening and testing: filling key data gaps. Toxicology 181–182, 371–382.

Grier, H.J., 1981. Cellular organization of the testis and spermatogenesis in fish. Am. Zool. 21, 345–357.

Grizzle, J., 1979. Anatomy and histology of the golden shiner and fathead minnow. PB-294-219, U.S. Dept. of Commerece, National Technical Information Service, Alabama Agricultural Experiment Station, Auburn, AL, USA.

Harries, J.E., Runnalls, T., Hill, E., Harris, C.A., Maddix, S., Sumpter, J.P., Tyler, C.R., 2000. Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). Environ. Sci. Technol. 34, 3003–3011.

Jensen, K.M., Korte, J.J., Kahl, M.D., Pasha, M.S., Ankley, G.T., 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 128, 127–141.

Jensen, K.M., Kahl, M.D., Makynen, E.A., Korte, J.J., Leino, R.L., Butterworth, B.C., Ankley, G.T., 2004. Characterization of responses to the antiandrogen flutamide in a short-term reproduction assay with the fathead minnow, Aquat. Toxicol., in press.

Kiernan, J.A., 1990. Histological & Histochemical Methods: Theory and Practice. Pergamon Press, Oxford, England, New York.

Kramer, V.J., Miles-Richardson, S., Pierens, S.L., Giesy, J.P., 1998. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to waterborne 17 beta-estradiol. Aquat. Toxicol. 40, 335–360.

Lange, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., Sumpter, J.P., 2001. Effects of the synthetic estrogen 17 alpha-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20, 1216–1227.

Leino, R.L., McCormick, J.H., 1997. Reproductive characteristics of the ruffe, *Gymnocephalus cernuus*, in the St. Louis River estuary on western Lake Superior: a histological examination of the ovaries over one annual cycle. Can. J. Fish. Aquat. Sci. 54, 256–263.

Leino, R.L., McCormick, J.H., Jensen, K.M., 1990. Multiple effects of acid and aluminum on brood stock and progeny of fathead minnows, with emphasis on histopathology. Can. J. Zool. 68, 234–244.

Makynen, E.A., Kahl, M.D., Jensen, K.M., Tietge, J.E., Wells, K.L., Van Der Kraak, G., Ankley, G.T., 2000. Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). Aquat. Toxicol. 48, 461–475.

McCormick, J.H., Stokes, G.N., Hermanutz, R.O., 1989. Oocyte atresia and reproductive success in fathead minnows (*Pimephales promelas*) exposed to acidified hardwater environments. Arch. Environ. Contam. Toxicol. 18, 207–214.

Miles-Richardson, S.R., Kramer, V.J., Fitzgerald, S.D., Render, J.A., Yamini, B., Barbee, S.J., Giesy, J.P., 1999a. Effects of waterborne exposure of 17 beta-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Aquat. Toxicol. 47, 129–145.

Miles-Richardson, S.R., Pierens, S.L., Nichols, K.M., Kramer, V.J., Snyder, E.M., Snyder, S.A., Render, J.A., Fitzgerald, S.D., Giesy, J.P., 1999b. Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Environ. Res. 80, 122–137.

Nagahama, Y., Chan, K., Hoar, W.S., 1976. Histochemistry and ultrastructure of pre- and post-ovulatory follicles in the ovary of the goldfish, *Carassius auratus*. Can. J. Zool. 54, 1128–1139.

Parrott, J.L., Wood, C.S., 2002. Fathead minnow lifecycle tests for detection of endocrine-disrupting substances in effluents. Water Qual. Res. J. Can. 37, 651–667.

Roberts, R.J., 1978. Fish Pathology. Balliere Tindall, London, England.

Selman, K., Wallace, R.A., 1986. Gametogenesis in *Fundulus heteroclitus*. Am. Zool. 26, 173–192.

Selman, K., Wallace Robin, A., Sarka, A., Qi, X., 1993. Stages of oocyte development in the zebrafish, *Brachydanio rerio*. J. Morp. 218, 203–224.

Shimizu, A., 1997. Reproductive cycles in a reared strain of the mummichog, a daily spawner. J. Fish Biol. 51, 724–737.

Smith, R.J.F., 1978. Seasonal changes in the histology of the gonads and dorsal skin of the fathead minnow, *Pimephales promelas*. Can. J. Zool. 56, 2103–2109.

USEPA, 1998. Endocrine disruptor screening and testing advisory committee (EDSTAC) report, OPPTS, Washington, DC.

USEPA, 2002. A short-term test method for assessing the reproductive toxicity of endocrine-disrupting chemicals using the fathead minnow (*Pimephales promelas*). EPA-600/R-01/067, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Duluth, MN, USA.

van den Belt, K., Wester, P.W., van der Ven, L.T., Verheyen, R., Witters, H., 2002. Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): time dependency and reversibility. Environ. Toxicol. Chem. 21, 767–775.

van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.

van der Ven, L.T., Wester, P.W., Vos, J.G., 2003. Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 22, 908–913.

Vos, J.G., Dybing, E., Greim, H.A., Ladefoged, O., Lambre, C., Tarazona, J.V., Brandt, I., Vethaak, A.D., 2000. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Crit. Rev. Toxicol. 30, 71–133.

Wester, P.W., van der Ven, L.T.M., Vethaak, A.D., Grinwis, G.C.M., Vos, J.G., 2002. Aquatic toxicology: opportunities for advancement through histopathology. Environ. Toxicol. Pharmacol. 11, 289–295.